

Iron Acquisition by *Ornithobacterium rhinotracheale*

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SUMMARY. *Ornithobacterium rhinotracheale* (ORT) is an emerging respiratory pathogen of poultry in North America that is causing millions of dollars in economic losses to the poultry industry. *Ornithobacterium rhinotracheale* is associated with airsacculitis, pleuritis, pneumonia, and consolidation of lungs. Little is known about the molecular mechanisms of infection. In this study, the mechanism of iron acquisition by *O. rhinotracheale* was explored. *O. rhinotracheale* strains grown under iron deprivation in media containing 200 μ M 2,2'-dipyridyl did not secrete siderophores as measured by the chrome azurol S (CAS) agar and CAS solution assays. Filter disks impregnated with various protein-bound iron compounds and inorganic iron salts of Fe(III) and Fe(II) placed on iron-restricted agar inoculated with a lawn of *O. rhinotracheale* supported growth from sheep and porcine hemoglobins, ovotransferrin, Fe(III), and Fe(II), but they did not support growth from bovine transferrin, bovine apo-transferrin, bovine lactoferrin, and hemin. However, both bovine hemoglobin and transferrin supported growth of *O. rhinotracheale* serotype C. Four immunoreactive proteins involved in iron acquisition were identified in an *O. rhinotracheale* membrane extract by using mass spectrometry. Furthermore, *O. rhinotracheale* field strains showed differential sensitivity to 2,2'-dipyridyl. Of the 72 field strains tested, 22 strains were resistant to the iron chelator at concentrations of 50 μ M and 100 μ M, suggesting this attribute may be related to disease-producing potential of these strains. This is the first report on the identification of the iron acquisition mechanism of *O. rhinotracheale*.

RESUMEN. Adquisición de hierro por parte del *Ornithobacterium rhinotracheale*.

El *Ornithobacterium rhinotracheale* es un patógeno respiratorio emergente en las aves domésticas de Norte América que está causando millones de dólares en pérdidas a la industria avícola. El *Ornithobacterium rhinotracheale* se asocia con aerosaculitis, pleuritis, neumonía y consolidación en los pulmones. Poco se conoce sobre los mecanismos moleculares de infección. En este estudio se exploraron los mecanismos de adquisición de hierro por parte del *Ornithobacterium rhinotracheale*. Según se determinó mediante pruebas de agar de cromo azurol-S y de solución de cromo azurol-S, las cepas de *Ornithobacterium rhinotracheale* cultivadas con privación de hierro en un medio con 200 μ M de 2,2'-dipiridil, no secretaron sideróforos. Discos impregnados con diferentes compuestos proteicos unidos a hierro y sales inorgánicas de hierro Fe (III) y Fe (II) colocadas en agar privado de hierro inoculado con una capa de *O. rhinotracheale*, permitieron crecimiento a partir de hemoglobinas de oveja y cerdo, de ovotransferrinas, Fe (III) y Fe (II), pero no a partir de transferrina bovina, apo-transferrina bovina, lactotransferrina bovina y hemina, Fe (III) y Fe (II). Sin embargo, tanto la hemoglobina como la transferrina bovina permitieron el crecimiento del *O. rhinotracheale* tipo C. En extractos de membrana de *O. rhinotracheale*, se detectaron cuatro proteínas inmunoreactivas relacionadas con el proceso de adquisición de hierro mediante espectrofotometría de masa. Además, las cepas de campo de *O. rhinotracheale* mostraron diferente sensibilidad al 2,2'-dipiridil. De las 72 cepas de campo evaluadas, 22 cepas resultaron resistentes al quelante de hierro en concentraciones de 50 a 100 μ M, sugiriendo que este atributo puede estar relacionado al potencial para producir enfermedad en estas cepas. Este es el primer reporte sobre la identificación del mecanismo de adquisición del hierro en *O. rhinotracheale*.

Key words: *Ornithobacterium rhinotracheale*, iron acquisition, outer membrane proteins

Abbreviations: 2-D = two-dimensional; BHI = brain heart infusion; CAS = chromazurol S; DP = 2,2'-dipyridyl; HEPES = *N*-(2-hydroxyethyl)piperazine-*N'*-(butanesulfonic acid); MALDI-TOF = matrix-assisted laser desorption ionization/time-of-flight; MS/MS = tandem mass spectrometry; ORT = *Ornithobacterium rhinotracheale*; PBS = phosphate-buffered saline; PVDF = polyvinylidene difluoride; SDS = sodium dodecyl sulfate; TMB = 3,3',5,5'-tetramethylbenzidine

Ornithobacterium rhinotracheale (ORT) is an emerging worldwide respiratory pathogen of turkeys and chicken, causing millions of dollars in economic losses to the poultry industry. In 1994, this bacterium was recognized as a separate genus consisting of one species (18). *Ornithobacterium rhinotracheale* is a slow growing gram-negative pleomorphic bacterium that does not require NAD (factor 10) nor hemin (factor 9) (18). It grows optimally at 7.5% to 10% CO₂, but it can also grow aerobically. To date, 18 serotypes, A through R, have been recognized (5). Serotype A, and to a lesser extent serotype B, is primarily isolated from turkeys in the United States. Symptoms include airsacculitis, pleuritis, and pneumonia,

and it is associated with consolidation of the lungs (5). *Ornithobacterium rhinotracheale* is also responsible for severe weight loss in meat turkeys and broilers, and it causes a drop in egg production in broilers (16).

Although no commercial vaccines are available in North America, autogenous vaccines have been used (Nagaraja, pers. obs.). An experimental peptide-based vaccine showed promise for controlling disease in chickens (13,14), and it is commercially produced in the Netherlands.

Ornithobacterium rhinotracheale may be considered an opportunistic pathogen, because infection often occurs after viral infection (8) or environmental stress. Both avian pneumovirus (8) and *Escherichia coli* are often isolated from upper respiratory tissues, along with *O. rhinotracheale*.

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Table 1. Bacterial strains of *O. rhinotracheale*.

Strain no.	Strain designation	Serotype	Tissue isolation	ID/origin
P5883		C		Strain #31, University of Minnesota
P5884		A		Strain #83, University of Minnesota
P5886		E		Strain #87, University of Minnesota
P5887		A	Lung/pneumonia	Strain #88, University of Minnesota
P5894		?	Respiratory tract	ATCC strain 51463, U.K.
DP-resistant field strains ^{A,B}				
2	P5902		Lung	3311-05, Arkansas
17	P5917		Unknown	05-573, California
21	P5921		Unknown	Ko500325, California
29	P5929		Unknown	97-8743, Georgia
31	P5931		Lung	H06-03631, North Carolina
32	P5932		Lung	H06-030791, North Carolina
33	P5933		Lung	H06-32456, North Carolina
34	P5934		Lung	R06-019458, North Carolina
38	P5938		Lung	North Carolina
41	P5941		Lung	D05-43244, Minnesota
44	P5944		Lung	D05-045394, Minnesota
45	P5945		Lung	D05-045749, Minnesota
48	P5948		Trachea	D05-061879
50	P5950		Trachea	D05-059109, Minnesota
52	P5952		Liver	D05-062700, Minnesota
53	P5953		Trachea	D05-064033, Minnesota
57	P5957		Lung	D06-001518, Minnesota
59	P5959		Sinus	D06-005405, Minnesota
60	P5960		Avian	D06-15694, Minnesota
61	P5961		Avian	D06-25204, Minnesota
64	P5964		Avian	D06-9946-1, Minnesota
66	P5966		Avian	D06-10761, Minnesota
67	P5967		Avian	D06-10652
69	P5969		Avian	D06-12314

^AStrains were resistant to DP; see Results.

^BRemaining 55 strains were sensitive to DP, did not grow in BHI broth, or both.

The molecular mechanism of infection by *O. rhinotracheale* is not understood. Iron acquisition is one of the mechanisms pathogens use to overcome the host's capacity to limit available iron during the infection process. Numerous iron acquisition systems have been described previously (19), including a Fur-independent, constitutive TonB-dependent mechanism (12); a Fur-dependent synthesis of low-molecular-weight iron chelators or siderophores (9,11), and Fur-dependent receptor/soluble receptor systems (3,7,10). It is not known which mechanism *O. rhinotracheale* uses to acquire iron necessary to infect and persist in its host.

The objective of this study was to identify the iron acquisition mechanism used by *O. rhinotracheale*.

MATERIALS AND METHODS

Organisms and culture. Characterized strains and field strains of *O. rhinotracheale* are shown in Table 1. *Ornithobacterium rhinotracheale* ATCC 51463 was obtained from the American Type Culture Collection (Manassas, VA). *Ornithobacterium rhinotracheale* strains P5883 (#31, serotype C), P5884 (#83, serotype A), P5886 (#87, serotype E), P5887 (#88, serotype A), and the field strains were from the culture collection of K. V. Nagaraja. Stock cultures were maintained frozen at -80°C in brain heart infusion (BHI) broth (BD Biosciences, Sparks, MD) containing 20% glycerol. A loopful of frozen culture was streaked on prewarmed blood agar (trypticase soy agar, 5% defibrinated sheep blood; BD Biosciences) and incubated for 48 hr at 37°C , 7.5% humidified CO_2 . To provide a broth stock culture for further studies, 10 to 15 1-mm to 2-mm colonies were removed and transferred into 5 ml of prewarmed BHI broth contained in a 14-ml Falcon sterile snap-cap tube (Falcon; BD Biosciences Discovery Labware, Franklin Lakes, NJ).

Alternatively, a prewetted swab of heavy growth was inoculated into 5 ml of prewarmed BHI broth. Cultures were then incubated for 16 hr at 37°C on an orbital shaker set at 200 rpm. One milliliter of culture was removed aseptically to obtain optical density values at 600 nm. Alternatively, 200 μl of culture was deposited in wells of a microtiter plate, and the optical density was measured at 600 nm with a plate reader (model 250, Molecular Devices, Sunnyvale, CA). The readings were multiplied by a factor of 1.43 ($10\text{ mm} \div 7\text{ mm}$) to convert to equivalent optical density readings based on a 10-mm pathlength.

Chemicals and biochemicals. All inorganic chemicals were of reagent grade purity. Chelex-100 was obtained from Bio-Rad (Hercules, CA). 2,2'-Dipyridyl, chrome azurol S, hexadecyltrimethylammonium chloride, bovine transferrin, bovine apo-transferrin, bovine lactoferrin, bovine hemoglobin, porcine hemoglobin, sheep hemoglobin, and ovotransferrin were obtained from Sigma Chemical Co. (St. Louis, MO). Porcine transferrin was obtained from First Link (Briarley Hill, U.K.). EZ-Link NHS-aminobiotin, horseradish peroxidase-labeled streptavidin, and Tween 80 were from Pierce Chemical (Rockford, IL). The 3,3',5,5'-tetramethylbenzidine (TMB) solutions were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Growth media containing 2,2'-dipyridyl (DP). BHI broth and BHI agar containing 200 μM DP was prepared by adding the appropriate volume of 25 mM sterile-filtered DP to the medium. CAS agar was prepared as described by Schwynn and Neilands (15), but using BHI agar containing 200 μM DP, because minimal medium such as Luria-Bertani broth medium containing agar did not support growth of *O. rhinotracheale*.

Effect of DP on growth. To examine tolerance of the *O. rhinotracheale* strains to DP, growth studies were carried out in a microtiter plate format. Wells containing 150 μl of sterile BHI broth containing from 0 to 400 μM final concentrations of DP were inoculated with 50 μl of a 24-hr culture adjusted to provide a final

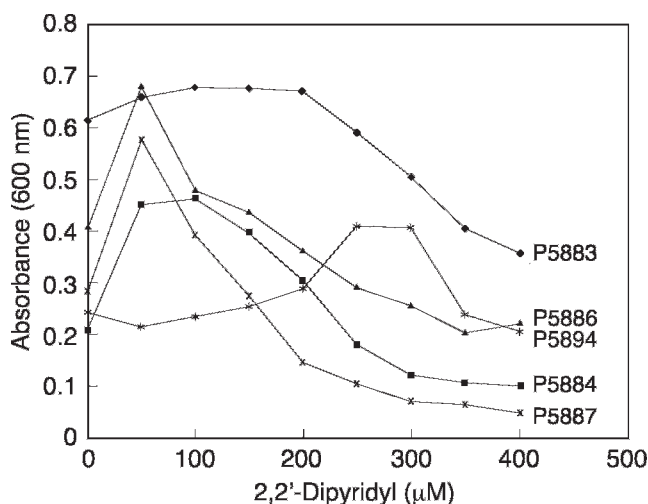


Fig. 1. Effect of DP on growth of five selected strains of *O. rhinotracheale*. Microtiter plates containing brain heart infusion broth adjusted to various concentrations of DP ranging from 0 μ M to 400 μ M were inoculated with *O. rhinotracheale* as described under Materials and Methods. Absorbance values at 600 nm were recorded after 24 hr of incubation under 7.5% CO₂ at 37 C. The strains are as follows: P5883 (#31, serotype C), P5884 (#83, serotype A), P5886 (#87, serotype E), P5887 (#88, serotype A), and P5894 (ATCC strain 51463).

A_{600} of 0.1. Cultures were incubated for 24 hr at 37 C and 7.5% humidified CO₂ on a microplate shaker (Lab-Line Instruments, Inc., Melrose Park, IL), set at 1.5. The average absorbances at 600 nm of duplicate cultures at each concentration of DP were measured and plotted against the concentration of DP.

Filter disk assay for iron uptake. A volume of 0.1 ml of a 10-fold dilution of a 16-hr culture adjusted to 0.5 absorbance units at 600 nm was spread on prewarmed plates of BHI agar containing 200 μ M DP. Sterile 10-mm Whatman no. 1 (Whatman, Florham Park, NJ) filter disks were placed on the surface of the agar. A volume of 30 μ l of a filter-sterilized 200 μ M iron source prepared in Chelex-100 (Bio-Rad)-treated deionized UltraPure water (Barnstead, Dubuque, IA) was deposited on the filter disk. Hemin was first dissolved in 0.01 N NaOH as a 10 mM solution, and then it was diluted to provide a solution containing 200 μ M in iron. Plates were incubated, agar surface facing up, at 37 C in 7.5% humidified CO₂. After 1 hr of incubation, plates were inverted, and then they were incubated up to 72 hr. Growth around the filter disks was recorded as positive or negative as follows: + indicates <3-mm zone of growth, ++ indicates 3-to-5-mm zone of growth, +++ indicates >5-mm zone of confluent growth, and - indicates no growth.

Siderophore production in broth cultures and CAS agar. Cultures grown in BHI broth in the absence or presence of 200 μ M DP for 24 hr or 48 hr were centrifuged at 10,000 \times g. A volume of 0.5 ml of supernatant was transferred to a cuvette, and 0.5 ml of CAS assay solution (15) was added. Change in absorbance at 680 nm was determined after 48 hr of incubation at room temperature. In addition, the assay solutions were scanned from 500 nm to 700 nm to confirm reduction in absorbance at 600 nm signifying iron removal from the CAS complex. Culture supernatant from *E. coli* strain ATCC 35218 and *E. coli* strain DH5 α served as positive and negative controls, respectively. In addition, BHI broth with and without DP served as medium controls.

Prewarmed CAS-BHI agar plates were streaked with a loop of culture obtained from one 2-mm colony grown on trypticase soy agar-blood agar plate. Plates were incubated for 48 hr at 37 C in a humidified 7.5% CO₂ atmosphere (for *O. rhinotracheale*), and then they were observed for the appearance of an orange-yellow zone around the streaked culture. *Escherichia coli* strain ATCC 35218 and *E. coli* DH5 α were streaked on the same plate 24 hr after streaking the *O. rhinotracheale* cultures. These

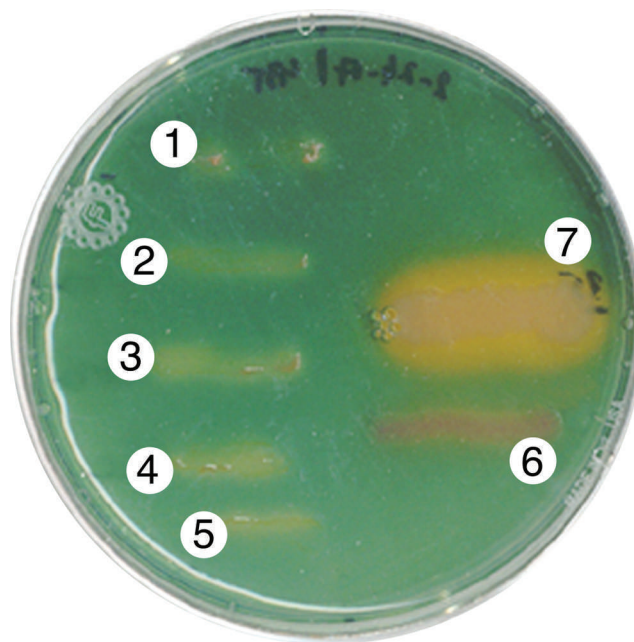


Fig. 2. CAS agar assay for production of siderophores by *O. rhinotracheale*. A loop-full of each of five strains of *O. rhinotracheale* grown 48 hr on a blood agar plate was streaked onto a CAS agar plate and incubated for 48 hr under 7.5% CO₂ at 37 C. Similarly, a loop-full of an overnight blood agar culture of the *E. coli* strains was streaked on the CAS agar plate the last 8 hr of incubation. Numbers indicate the following: 1, P5883 (serotype C); 2, P5884 (serotype A); 3, P5886 (serotype E); 4, P5887 (serotype A); 5, P5894 (ATCC strain 51463); 6, *E. coli* strain DH5 α ; and 7, *E. coli* strain ATCC 35218.

strains served as positive and negative controls, respectively. Growing *E. coli* under CO₂ had no effect on the outcome of the results. *Escherichia coli* DH5 α served as a negative control because this phage T1 deletion mutant (Invitrogen, Carlsbad, CA) is reportedly negative for the ferric siderophore receptor and siderophore production (2,4,6); this was confirmed by testing the strain on CAS agar.

Two-dimensional (2-D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. Samples for 2-D electrophoresis were prepared as follows. Cultures (100 ml) grown in BHI broth for 48 hr at 37 C with agitation (150 rpm) were centrifuged at 7500 \times g. The cell pellet was washed with 0.1 M phosphate-buffered saline (PBS), pH 7.2, and resuspended in a small volume (0.5 ml) of 2.5% NaCl in 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(butanesulfonic acid) (HEPES), pH 7.4, and heated for 1 hr at 56 C to extract the outer membrane proteins (1). The extracted protein was dialyzed against 20 mM HEPES, pH 7.4. Protein concentration was determined using the bicinchoninic acid assay (Pierce Chemical). One hundred micrograms of protein in 50 μ l was mixed with 100 μ l of rehydration buffer (GE Healthcare Amersham Pharmacia Biotech, Piscataway, NJ) and applied to the Zoom IEF strip (pH 3–10 NL; Invitrogen). Isoelectric focusing was performed using Zoom IPGRunner (Invitrogen) according to manufacturer's direction. The IEF strip was removed, equilibrated in SDS-reducing buffer (GE Healthcare Amersham Pharmacia Biotech), and applied to the NuPAGE Zoom Bis-Tris 4–12% gradient gel (Invitrogen). After 2-D electrophoresis for 183 V-hr, the gel was either stained (with 0.1% Coomassie Blue R-250 in 40% [v/v] methanol, 7.5% [v/v] acetic acid and destained in 40% methanol, 7.5% acetic acid) or prepared for Western blotting using XCell Surelock blotting module (Invitrogen). The electrotransfer to polyvinylidene difluoride (PVDF) was performed in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0, at 4 C to 60 V-hr. After transfer, the PVDF was submerged in methanol, allowed to air dry, and incubated with hyperimmune chicken serum to *O. rhinotracheale* diluted 1:5000 in

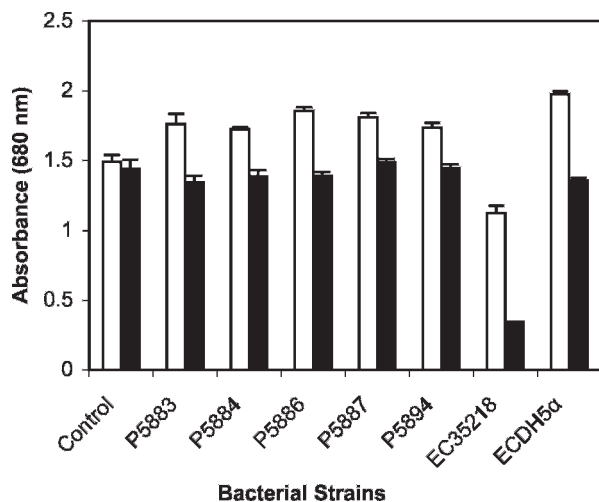


Fig. 3. CAS assay for siderophore production by *O. rhinotracheale* strains and *E. coli*. Five strains of *O. rhinotracheale*, P5883, P5884, P5886, P5887, and P5894, and two strains of *E. coli*, ATCC 35218 and DH5 α , were tested in triplicate for the production of siderophores after 24 hr of growth in BHI broth in the presence and absence of 200 μ M DP. *Escherichia coli* ATCC 35218 (EC35218) served as a positive control; *E. coli* DH5 α (ECDH5 α) served as a negative control. Control, BHI broth without (white bars) and with (black bars) DP. Standard deviations of three replicate determinations are indicated on the bars. A decrease in absorbance at 680 nm of the CAS-iron complex indicates removal of iron from the complex.

0.1 M PBS, pH 7.4, and 0.05% Tween 80 containing 0.25% fish gelatin. The blot was subjected to rapid immunodetection according to manufacturer's direction (Millipore Corporation, Billerica, MA). The conjugate, horseradish peroxidase labeled goat-anti-chicken IgG, was diluted 1:5000 in the same buffer. Blots were developed using the TMB substrate system (Kirkegaard and Perry Laboratories).

For MALDI-TOF analysis, gel plugs were removed from the stained 2-D gel and digested with trypsin as described previously (17). Briefly, gel plugs were removed, digested on an automated digester, and subjected to mass spectrometric analysis using a QStar XL mass spectrometer (Applied Biosystems, Foster City, CA) located at the Iowa State University Plant Science Institute Proteomics Facility (Ames, IA).

The peptide mass spectra were analyzed using the Prospector software utility MS-FIT available at <http://www.ucsf.edu>. De novo sequences were obtained using Analyst QS and Bioanalyst v1.1 (Applied Biosystems).

Statistical analysis. The culture absorbance data were analyzed using the Prism 4 (GraphPad Software Inc, San Diego, CA) statistical analysis program.

RESULTS

Effect of DP on growth of *O. rhinotracheale*. The iron chelator DP was added to the growth medium to render the medium iron restricted and to mimic the host's environment for the invading pathogen. It was of interest to determine whether the five characterized strains exhibited different sensitivities to DP by using various concentrations of the iron chelator. The results of five characterized strains of *O. rhinotracheale* grown in microtiter plates in BHI broth containing various concentrations of DP are shown in Fig. 1. Each data point represents the average absorbance of duplicate cultures at 600 nm after 24 hr of incubation. The results indicate that strain P5883 is less sensitive to increasing DP concentrations than strains P5884, P5886, and P5887. Strain P5894 seemed to be tolerant to DP over the concentration range studied. A concentration of 200 μ M DP was subsequently used in all iron-restricted media in this study.

Growth of *O. rhinotracheale* strains in CAS supplemented media. The universal CAS assay was developed by Schwynn and Neilands (15) to determine whether *O. rhinotracheale* secreted siderophores to acquire iron. As shown in Fig. 2, the five strains of *O. rhinotracheale* did not secrete siderophores when grown on CAS agar. Nor were siderophores detected when these strains were grown under iron-restricted conditions (Fig. 3) in BHI broth and tested with the CAS assay reagent (15). Fig. 2 shows the result of ORT strains cultured on CAS agar. Only very narrow yellow zones were observed around the culture streak of the five *O. rhinotracheale* strains. In contrast, the *E. coli* siderophore-producing strain ATCC 35218 showed a large yellow-orange halo. The *E. coli* DH5 α strain that is negative for siderophore production showed a very narrow yellow zone around the bacterial growth.

Table 2. Iron sources for *O. rhinotracheale* serotype A and C.^A

Iron source	P5884, serotype A				P5883, serotype C			
	24 hr		48 hr		24 hr		48 hr	
	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²
Bovine albumin	— ^B	—	—	—	—	—	—	—
Bovine holo-transferrin	—	—	—	—	—	—	—	+ ^C
Bovine apo-transferrin	—	—	—	—	—	—	—	—
Bovine lactoferrin	—	—	—	—	—	—	—	—
Ovotransferrin	+	ND ^D	+	ND	+	+	+++	+++
Bovine hemoglobin	—	ND	—	ND	+	+	+++	+++
Porcine hemoglobin	+	+	++	++	+	+	++	++
Sheep hemoglobin	+	+	+	+++	+	+	+++	+++
Ferric ammonium sulfate	+	+	++	++	+	+	++	++
Ferric citrate	+	+	++	++	+	+	++	++
Ferrous sulfate	++	++	+++	+++	++	++	+++	+++
Hemin	—	—	—	—	—	—	—	—

^ABHI agar plates containing 200 μ M DP were inoculated with a lawn of either a 10-fold or 100-fold dilution of a 48-hr culture of *O. rhinotracheale* in BHI broth without DP. Duplicate experiments were performed.

^BDash indicates no growth was observed.

^CPositive sign indicates growth was observed around the edges of the filter disk; +, <3 mm zone of growth; ++, 3-to-5-mm zone of growth; +++, >5 mm zone of confluent growth.

^DND = not done.

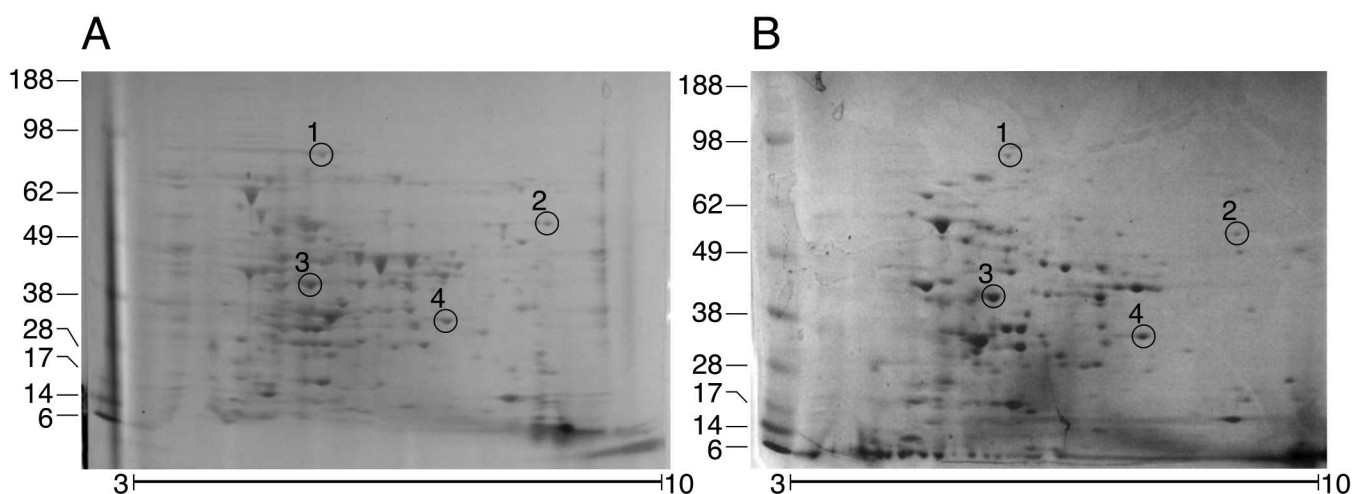


Fig. 4. 2-D gels of *O. rhinotracheale* strains P5883 and P5884. (A and B) Gels stained with Coomassie Brilliant Blue. (A) Strain P5883 (serotype C). (B) Strain P5884 (serotype A). Spot 1, Fep A homolog; spot 2, heme-hemopexin-binding protein; spot 3, iron-binding protein; and spot 4, TonB-related protein. Molecular weight markers are in kilodaltons; pI gradient is from pI 3 to pI 10.0, as indicated by the line under the gels.

CAS assay results of culture supernatants of bacterial strains grown in iron-restricted BHI broth showed similar results compared with the CAS agar assay. The percentage of decreases in absorbance for *O. rhinotracheale* strains P5883, P5884, P5886, P5887, P5894, and *E. coli* DH5 α (Fig. 3, black bars) compared with the control medium (Fig. 3, black bars) were 6.8, 4.0, 4.0, 3.6, -3.0, -0.2, and 5.9%, respectively. A major decrease in absorbance of 76.3% was observed for *E. coli* ATCC 35218 compared with the same control medium. The increase in absorbance for CAS assays of *O. rhinotracheale* strains and *E. coli* DH5 α (Fig. 3, white bars) indicates lack of siderophore production compared with the control medium. A decrease in absorbance is observed for *E. coli* ATCC 35218 grown in the absence of DP, which is thought to be due to Krebs cycle metabolites (15). The slight decrease observed with the assay results of the siderophore-producing *E. coli* strain. The siderophore-negative *E. coli* strain DH5 α also showed a decrease in the maximum absorbance at 600 nm of the CAS complex, but to a much lesser extent than the siderophore-producing *E. coli* strain.

Iron sources for growth of *O. rhinotracheale*. Various organic and inorganic iron compounds applied to a sterile filter disk (containing 6 μ mol of iron per disk) were tested as a source of iron for *O. rhinotracheale*. The results of growth of *O. rhinotracheale*

around the sterile filter disks impregnated with various iron sources are shown in Table 2. Bovine serum albumin and apo-transferrin served as controls. The results indicated that strain P5884 (serotype A) and strain P5883 (serotype C) used iron from sheep and porcine hemoglobin, from ovotransferrin, and from ferric and ferrous iron compounds. In addition, serotype C used iron from bovine hemoglobin and holo-transferrin. Bovine serum albumin, bovine apo-transferrin, bovine lactoferrin, and hemin did not support growth of *O. rhinotracheale* serotypes A and C.

Identification of immunoreactive proteins involved in iron acquisition. Western blots developed with hyperimmune chicken serum (data not shown) were used to identify the corresponding proteins on the Coomassie Blue-stained gels (Fig. 4). These spots were then excised and submitted for tandem mass spectrometric (MS/MS) sequencing. Four of the 17 proteins identified with the serum, marked 1 through 4 (Fig. 4), were related to iron acquisition (Table 3). These proteins are 1) a 105-kDa protein homologous to hypothetical outer membrane receptor protein of *Haemophilus influenzae* (accession no. P45182) that shares homology with ferrienterochelin and the colicin binding sequences; 2) a 58-kDa protein homologous to the heme-hemopexin-binding protein B of *H. influenzae* (accession

Table 3. *O. rhinotracheale* protein identification by *de novo* MS/MS sequencing.^A

Protein spot	Homology	Mol mass (kDa)	Peptide sequence ^B	Accession no.
1 ^C	FepA homolog	105	— ^C	P05825
2	Heme-hemopexin-binding protein	85	GQDLN RFRMDPS QLRNBRF ^D	P45356
3	Iron-binding protein	36	EMATA EQWW RYSHLVS	Q57449
4	TonB-dependent membrane receptor	32	LQOYGN ^D TLGBFOQ ^D	120437375

^AProtein spots were identified from a blot of a 2-D gel of outer membrane protein extracts prepared from *O. rhinotracheale* P5883 and P5884 (see Materials and Methods) treated with hyperimmune chicken serum. Four of the 17 spots identified were related to iron acquisition.

^BPeptide sequences are from *O. rhinotracheale* P5883. The sequences are from collision-induced parent ion fragments of the tryptic peptides.

^CProtein was identified by MALDI mass spectrometry peptide mass fingerprinting.

^DAmino acid residues B and O refer to carboxamidomethyl cysteine and oxidized methionine, respectively (Bioanalyst version 1.1, Data Dictionary; Applied Biosystems).

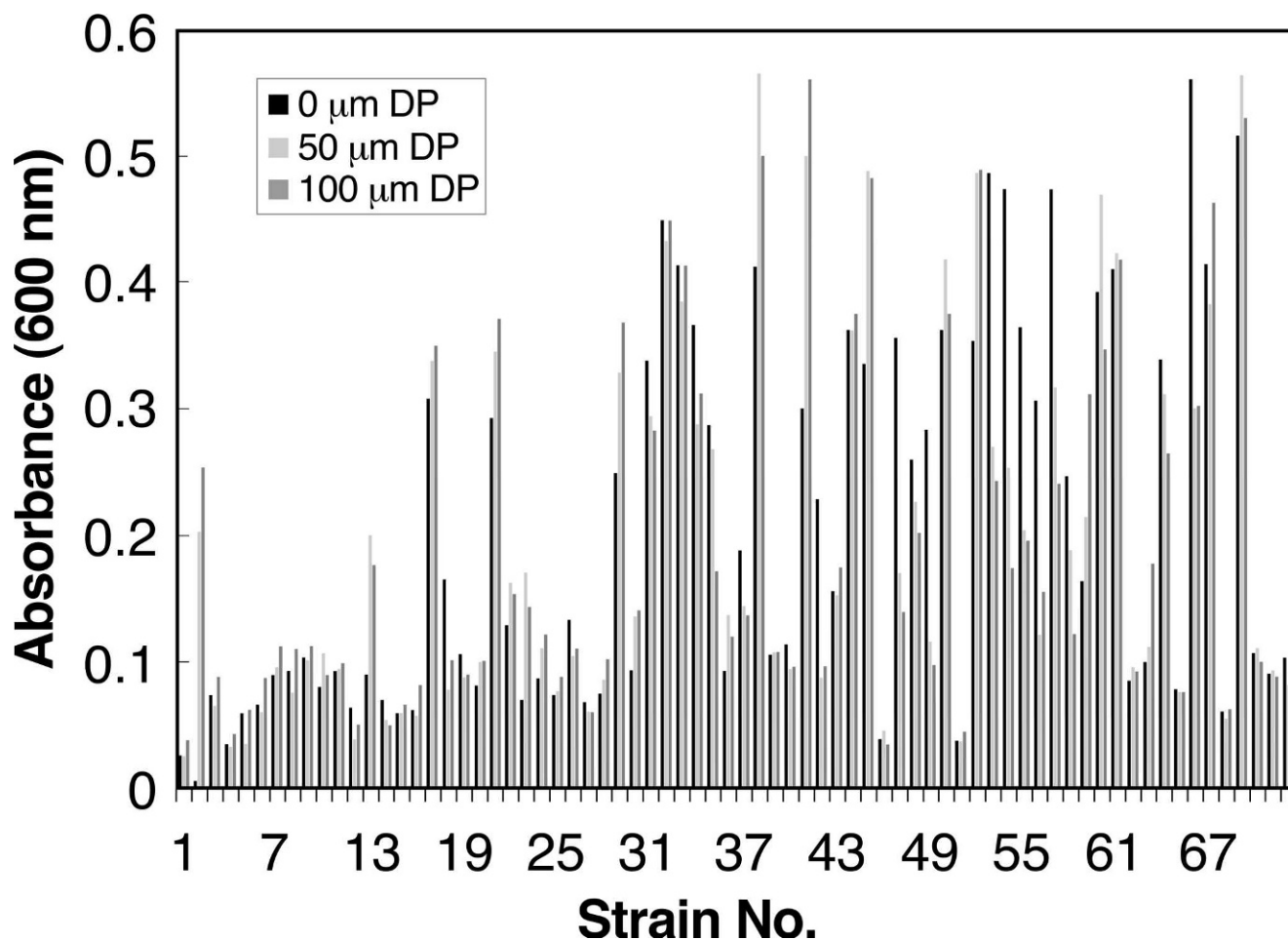


Fig. 5. Sensitivity to DP of *O. rhinotracheale* field strains. Duplicate cultures of each of the field strains were grown for 24 hr in the microtiter plate assay as described under Materials and Methods. Average absorbance values of duplicate cultures were recorded at 0 μ M, 50 μ M, and 100 μ M DP. The y-axis indicates absorbance values at 600 nm.

no. P45356); 3) a 36-kDa protein homologous to an iron-binding protein of *H. influenzae* (accession no. P57449); and 4) a 32-kDa protein homologous to a TonB receptor protein involved in iron transport from *Gramella forsetii* KT083, a flavobacterium (accession no. YP_863061.1). The corresponding peptide sequences from *Ornithobacterium* are shown in Table 3.

Sensitivity to DP. Because the five characterized strains, P5883, P5884, P5886, P5887, and P5894, differed in the concentration of DP required to reduce growth by 50%, it was of interest to test many field isolates of *O. rhinotracheale* for their sensitivity toward DP. Seventy-two strains were cultured in duplicate in the microtiter plate growth assay using DP concentrations ranging from 0 to 400 μ M. The average absorbance values of duplicate cultures at 0, 50, and 100 μ M DP are plotted in Fig. 5. Twenty-two of the 72 field isolates of *O. rhinotracheale* seemed to be “resistant” to DP at concentrations of 50 μ M and 100 μ M. The remaining strains were either sensitive or failed to grow in the medium, even when no DP was present. Growth did occur, however, on the blood agar plates from which the initial inoculum was prepared. Significant differences were observed between “sensitive” and resistant strains at $P < 0.05$ at the cut-off value of 0.230 at 600 nm for cultures tested at 50 μ M and 100 μ M DP (Fig. 6). Growth of the resistant strains is significant ($P < 0.05$) at an absorbance value of 0.250 at 600 nm or greater compared with the sensitive strains.

DISCUSSION

Ornithobacterium rhinotracheale is an emerging pathogen of poultry that causes considerable losses to the poultry industry (5). Very little is known about the virulence factors and virulence mechanisms of this pathogen; therefore, examination of the mechanism of iron acquisition might provide insight on the pathogenesis of this organism. Several *O. rhinotracheale* field strains of known serotype, an ATCC type strain, and 72 field strains of unknown serotype were examined for their ability to grow in BHI broth that was rendered iron restricted by using the iron chelator DP. First, the effect of iron-restricted media on the proliferation of *O. rhinotracheale* (Fig. 1) was examined. The different strains showed different responses to DP. Two of the strains, P5883 and P5894 (serotype C and ATCC 51463, respectively), tolerated higher concentrations of DP than strains P5884 and P5887 (both serotype A) and P5886 (serotype E). Because of observed differences in sensitivity of the strains to DP, the DP sensitivity studies were extended to include several field strains. Interestingly, a wide range in sensitivity was observed among the field strains (Fig. 4). Twenty-two of the 72 strains grew well in 50 μ M and 100 μ M DP. The remaining strains did not grow well in the BHI broth with or without DP. Multiple sequential subcultures did not improve growth characteristics of these strains in BHI broth. Supplementa-

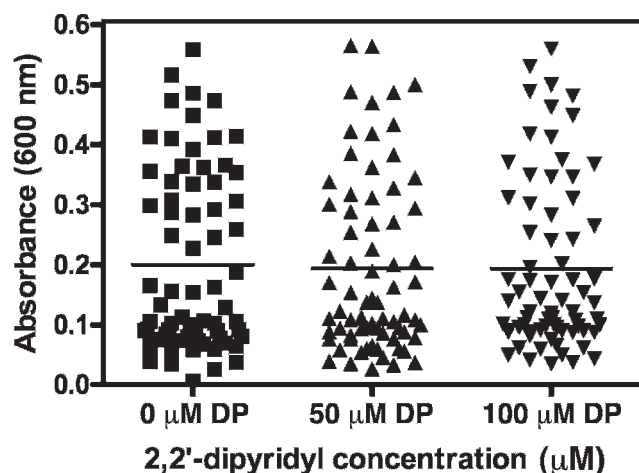


Fig. 6. Statistical analysis of the absorbance data at 600 nm of the *O. rhinotracheale* field strains grown at 0 μ M, 50 μ M, and 100 μ M DP. The horizontal line indicates the mean of the data at each concentration of DP. Growth of the resistant strains is significant ($P < 0.05$) at an absorbance value of 0.250 at 600 nm or greater compared with the sensitive strains.

tion of the growth medium with organic iron source such as sheep hemoglobin to improve growth of these particular field strains was not attempted for this study. The data showed, however, that there is a significant difference ($P < 0.05$) between resistant strains and the strains that grow poorly in BHI broth without DP. The molecular basis for this difference in growth of these field strains is not known. Studies are currently underway using comparative proteomics methodologies to determine quantitative differences in protein expression of the poorly growing strains and the resistant strains. The ability of 22 of the field strains of *O. rhinotracheale* to grow in the presence of DP suggested to us that this attribute may be related to disease-producing potential of these strains. These strains will be further tested in turkey poults.

To determine whether *O. rhinotracheale* produces siderophores as a mechanism to acquire iron, the protocols developed by Schwyn and Neilands (15) were used. The results shown in Figs. 2 and 3 support the conclusion that *O. rhinotracheale* strains do not produce siderophores. The alternative mechanism for iron acquisition is through the expression of one or more iron-protein receptor-mediated uptake systems (7,11). Iron use experiments with iron-protein sources revealed that sheep and bovine hemoglobin, bovine transferrin, and ovotransferrin supported growth of *O. rhinotracheale*, suggesting that the organism expressed a receptor for binding and acquiring iron from the carrier proteins. Although a hemoglobin receptor protein has not yet been identified, four outer membrane proteins that are involved in iron acquisition were identified by mass spectrometry of protein spots obtained from 2-D gels. Interestingly, hemin did not serve as a source of iron, suggesting that a heme receptor similar to BhuR of *Bordetella bronchiseptica* (10) was not expressed by *O. rhinotracheale*.

In summary, this study demonstrated that the mechanism of iron acquisition for *O. rhinotracheale* under iron-restricted conditions occurs via the iron-bound protein pathway rather than through the siderophore secretion pathway. This study also showed that many field strains had different sensitivities to the iron chelator DP. Surprisingly, only about one third of the isolates tested were resistant to DP, suggesting that these strains may potentially be more virulent. This is the first report describing the iron uptake mechanism for *O. rhinotracheale*.

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